**American Journal of Plant Sciences, 2015, 6, 811-817**

http://dx.doi.org/10.4236/ajps.2015.66087

# Soybean Seed Co-Inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A New Biotechnological Tool to Improve Yield and Sustainability

**Mariangela Hungria[1*], Marco Antonio Nogueira[1], Ricardo Silva Araujo[2]**

[1]Embrapa Soja, Londrina, Brazil
[2]Total Biotecnologia Indústria e Comércio Ltda, Curitiba, Brazil
Email: *mariangela.hungria@embrapa.br, *hungria@pq.cnpq.br, *biotecnologia.solo@hotmail.com, marco.nogueira@embrapa.br, rsaraujo@totalbiotecnologia.com.br

Received 10 February 2015; accepted 30 March 2015; published 3 April 2015

## Abstract

**Legume nodulation by rhizobia can supply crops with nitrogen and reduce environmental impacts caused by chemical fertilization. The soybean crop in Brazil is an impressive example of how biological $N_2$ fixation can be employed with a plant species of high economic value. However, the development of more productive cultivars, along with the increasing global climatic changes demand agricultural practices to become more productive and yet more environmentally friendly. Plant growth-promoting rhizobacteria (PGPR) are highly beneficial to agriculture worldwide, acting in plant nutrition, protection, and growth stimulation. *Azospirillum* is, certainly, the most employed PGPR in the world, but little is known about its interaction with rhizobia, when both are applied to legume seeds. We have evaluated the co-inoculation of bradyrhizobia and azospirilla on soybean seeds under different soil and climate conditions in Brazil. Our results demonstrated that co-inoculation is efficient and beneficial to the crop, and promotes yield increases without adding any chemical N fertilizers even in soils where established populations of soybean bradyrhizobia exist. The strategy of co-inoculation thus represents a new biotechnological tool to improve soybean yield without adding any chemical N fertilizers, thus contributing to current practices of sustainability in agriculture.**

## Keywords

**Soybean, *Azospirillum*, *Bradyrhizobium*, Inoculation, Yield**

---

[*]Corresponding author.

**How to cite this paper:** Hungria, M., Nogueira, M.A. and Araujo, R.S. (2015) Soybean Seed Co-Inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A New Biotechnological Tool to Improve Yield and Sustainability. *American Journal of Plant Sciences*, **6**, 811-817. http://dx.doi.org/10.4236/ajps.2015.66087

## 1. Introduction

The symbiosis between $N_2$-fixing bacteria and plants of the leguminous family has long been exploited in agriculture all over the world. Such bacteria capture atmospheric nitrogen, which is enzymatically reduced to ammonia inside root nodules, and then assimilated by plant tissues, in the form of nitrogenous compounds. In Brazil, the success of the soybean [*Glycine max* (L.) Merr.] as a cash crop is highly attributed to the benefits obtained from inoculating seeds with elite strains of *Bradyrhizobium japonicum*, *B. elkanii*, and *B. diazoefficiens*, a partnership that is capable of completely meeting the crop's nitrogen requirement to sustain high yields [1] [2].

Other beneficial soil microorganisms such as plant growth-promoting rhizobacteria (PGPR) have also been exploited in agriculture. PGPR perform an array of biological processes which are beneficial to plants, such as production of plant-growth hormones, like auxins [3], gibberellins [4], cytokines [3] [5], and ethylene [5], induction of plant resistance to stresses and diseases [6], phosphate solubilization [7], and also $N_2$ fixation [8]. Among such bacteria, those belonging to the genus *Azospirillum* are outstanding PGPR, having long been employed worldwide as plant inoculants, and, more recently, in Brazil too [9] [10].

The annual fixation of over 300 kg of N/ha, followed by the delivery of 20 to 30 kg of N/ha to the soil by the association between the soybeans and $N_2$-fixing bacteria are fundamental for the success of the crop in Brazil [1] [2]. However, the continuous development of soybean cultivars, aiming at higher yields, demands an increased supply of N for the crop, which, in turn, implies that investigation must be continued to assure the benefits of $N_2$ fixation to supply the crop. In addition, longer periods of drought and high temperatures, due to the global weather changes, which also affect Brazil, are a threat to the contribution of the biological processes to agriculture. It is known that plants subject to environmental stresses become more susceptible, for example, to pests and diseases, resulting in increased seed treatment with chemicals such as fungicides and insecticides before sowing. This practice, which is a reality for over 90% of the soybean seeds grown in Brazil [1] [11], may be very deleterious to seed-applied bacteria.

Given the importance of the soybean crop to Brazil, and the awareness of current and potential future limitations to biological $N_2$ fixation by *Bradyrhizobium*-inoculated soybeans, as well as the alleged benefits of inoculating different crops with *Azospirillum*, co-inoculation of soybeans with both bacteria sounds like an interesting and promising technology to improve the crop's performance. In addition, such approach fulfills current paradigms of agricultural, economic, social, and environmental sustainability. One recent study has addressed this issue for the soybean and common bean (*Phaseolus vulgaris* L.) crops [12], but in that case, *Bradyrhizobium* and *Rhizobium* were applied to the seeds, whereas *Azospirillum* was inoculated in the planting furrow. Although positive results were obtained, such practice may be of little interest to farmers because it increases planting labor. Therefore, we expanded our studies and here we report the results obtained when both *Bradyrhizobium* spp. and *Azospirillum brasilense* were applied to the soybean seeds at sowing.

## 2. Materials and Methods

Experiments were planted in the field in the 2010/2011 and 2012/2013 cropping seasons. In 2010/2011 trials were planted at two locations in the State of Paraná, Brazil, namely Londrina (23˚11'S, 51˚11'W, 620 m altitude, Köpen-Geiger climate type Cfa), and Ponta Grossa (25˚13'S, 50˚1'W, 880 m altitude, Köpen-Geiger climate type Cfb), both with previously established and naturalized populations of *Bradyrhizobium* in the soil. In 2012/2013 trials were set at two locations in the State of Goiás, Brazil, namely Rio Verde (17˚47'S; 50˚54'W, 730 m altitude, Köppen-Geiger climate type Aw) and CachoeiraDourada (18˚29'S; 49˚28'W, 450 m altitude, Köppen-Geiger climate type Aw), in soils devoid of naturalized bradyrhizobia.

At each location, 40 days before sowing, soil samples (0 - 20 cm) were collected for analysis of chemical, physical, and microbiological properties, as described before [2]. Soil rhizobial populations at each location (**Table 1**) were estimated by the most probable number (MPN, with soybean cultivar BMX Potência RR) technique, also as described before [2]. For the estimation of the populations of free-living diazotrophic bacteria, bacteria were count in NFb semi-solid medium [13].

In the 2010/11 crop season, in both Londrina and Ponta Grossa the soybean cultivar used was BRS 133 (conventional), while in 2012/13 BMX-Potência (RR) was used in Rio Verde and BRS-GO-8360 (conventional) in Cachoeira Dourada.

Inoculation treatments comprised either inoculation with *Bradyrhizobium* spp. alone (I) or co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense* (I + Azo). All inoculants employed were liquid commercial

**Table 1.** Soil chemical properties, texture and population of soybean bradyrhizobia at the 0 - 20 cm layer of the field sites where the experiments were performed. Analyses performed before sowing.

| Site | Year | pH | H + Al | K | Ca + Mg | P | C | SB$^a$ | BS$^a$ | Granulometry | | | *Bradyrhizobium* cells | Free-living diazotrophic Cells |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ($CaCl_2$) | ------- $cmol_c/dm^3$ ------- | | | $mg/dm^3$ | $g/dm^3$ | $cmol_c/dm^3$ | % | Clay | Silt | Sand | Cells/g soil | UFC/g soil |
| Londrina | 2010/11 | 5.35 | 4.18 | 0.43 | 5.62 | 7.77 | 21.68 | 6.05 | 59.11 | 71.0 | 8.2 | 20.8 | $1.79 \times 10^4$ | $9.0 \times 10^6$ |
| Ponta Grossa | 2010/11 | 4.89 | 4.75 | 0.23 | 4.85 | 2.83 | 25.73 | 5.08 | 68.84 | 23.8 | 30.0 | 73.2 | $2.14 \times 10^4$ | $7.5 \times 10^6$ |
| Rio Verde | 2012/13 | 5.14 | 3.64 | 0.80 | 3.43 | 9.56 | 22.55 | 4.23 | 53.75 | 36.3 | 9.6 | 54.1 | zero | $7.0 \times 10^6$ |
| Cachoeira Dourada | 2012/13 | 5.40 | 3.07 | 0.37 | 5.28 | 1.71 | 18.55 | 5.65 | 64.79 | 57.8 | 18.2 | 24,0 | 3.57 | $9.5 \times 10^5$ |

$^a$SB, sum of bases; BS, bases saturation = $[(K + Ca + Mg)/T_{cec}] \times 100$, where $T_{cec}$ = K + Ca + Mg + total acidity at pH 7.0 (H + Al).

products available in Brazil. Inoculant concentrations were determined by spread-plating on yeast-manitol-agar medium, in the case of *Bradyrhizobium*, whereas *Azospirillum* was counted by spread-plating on NFb [13] and RC [14] solid media. The concentration of *Bradyrhizobium* in the inoculant was estimated at $2.60 \times 10^{10}$ CFU/mL in 2010/11, and at $7.33 \times 10^9$ CFU/mL in 2012/13. The concentration of *Azospirillum* in the inoculant was $2.47 \times 10^8$ CFU/mL in 2010/2011 and $2.97 \times 10^8$ CFU/mL in 2012/2013. Both products were in accordance with Brazilian regulations [15], which require $>10^9$ cells/g or mL, in the case of *Bradyrhizobium*, and $>10^8$ cells/g or mL, in the case of *Azospirillum*. *Bradyrhizobium* inoculant contained strains SEMIA 5079 (*B. japonicum*) and SEMIA 5080 (*B. diazoefficiens*), and was applied to seeds before planting at an amount necessary to provide $1.2 \times 10^6$ cells of *Bradyrhizobium*/seed. The *A. brasilense* inoculant contained strains Ab-V5 and Ab-V6, and was applied to the seeds to provide $1.2 \times 10^5$ cells/seed. Seed inoculation was performed by mixing both inoculants with the seeds and leaving it to dry in the shadow for 1 h.

Two control treatments were included in each experiment. The first was a non-inoculated (NI) control, and the second was a NI control that received N fertilizer (NI + N). N fertilizer was applied as urea (46.6% N), at 200 kg N/ha, split in two applications of 50% each, one at sowing and the other at the R2 growth stage (fully open flower below one of the uppermost nodes of the main stem with a fully developed leaf).

Experiments were set in a completely randomized block design, with six replicates, and plots measured approximately 24 m$^2$. All plots were separated by 0.5 m-long rows, and 1.5 m-wide terraces to avoid cross contamination due to surface run-off, which might carry bacteria and or fertilizers. At all locations, base fertility was corrected by adding 300 kg/ha of a 0-28-20 NPK fertilizer. No N fertilizer was applied, except for the NI + N treatment plots. During the V4 vegetative growth phase (four nodes of the main stem above the unifoliate node with fully developed leaves) plants received foliar sprays of 20 g Mo/ha and 2 g Co/ha. Weeds were controlled with herbicides and pest control was accomplished by the utilization of both chemical and biological insecticides. Final plant population was of about 360,000 plants/ha.

Thirty-five to 50 days after sowing (DAS), five plants were collected from each plot for evaluation of nodulation (number and biomass), plant biomass, N content, and total N accumulated in the shoots, as described before [2]. Grain yield was determined at the end of the crop's cycle, from a central portion of each plot of (5.6 to 8 m$^2$). Seeds were cleaned and weighed, and grain yield was estimated after correction of seed weights to 13% moisture.

All data were submitted to tests that assess variable normality and variance homogeneity, followed by an analysis of variance (ANOVA). Duncan's test was employed to compare means in cases where statistical significance had been detected by the ANOVA F test. For all analyses, the Statistica version 7.0 software was employed.

## 3. Results

In Londrina, in the first season, neither inoculation nor co-inoculation resulted in significant increases in nodulation, whereas in Ponta Grossa, even though the soil had $2.14 \times 10^4$ cells of soybean bradyrhizobia/g soil, both treatments significantly improved nodule number and dry weight relative to the non-inoculated control (**Table 2**). It was interesting to notice that although co-inoculation did not increase nodulation relative to inoculation

**Table 2.** Nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), total shoot N content (TSNC) at 35 days after sowing (DAS) in Londrina and at 40 DAS in Ponta Grossa, and grain yield (Yield) of soybean plants (BRS 133, conventional), in response to seed inoculation consisting of single inoculation with *Bradyrhizobium*, or of co-inoculation with *Bradyrhizobium* and *Azospirillum*. Experiments performed in 2010/11 in soils containing $>10^4$ cells of soybean bradyrhizobia/g soil.

| Treatment* | Londrina | | | | | Ponta Grossa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NN | NDW | SDW | TSNC | Yield | NN | NDW | SDW | TSNC | Yield |
| | (n°/pl) | (mg/pl) | (g/pl) | (mg N/pl) | (kg/ha) | (n°/pl) | (mg/pl) | (g/pl) | (mg N/pl) | (kg/ha) |
| Non-inoculated | 32.1 a[a] | 112 a | 3.35 b | 107 b | 3360 c | 20.2 b | 56 b | 4.98 b | 145 b | 2599 c |
| Non-inoculated + N-fertilizer (200 kg N/ha) | 18.9 b | 24 b | 4.01 a | 134 a | 3760 a | 17.7 b | 44 b | 6.88 a | 223 a | 3069 a |
| I (inoculated with *Bradyrhizobium*) | 33.2 a | 123 a | 4.08 a | 136 a | 3512 b | 42.1 a | 195 a | 6.96 a | 188 ab | 2877 b |
| I + Azo (co-inoculated with *Bradyrhizobium* and *Azospirillum*) | 35.6 a | 120 a | 3.88 a | 129 ab | 3755 a | 38.8 a | 181 a | 6.55 a | 218 a | 3044 a |

[a]Means (n = 6) followed by different letters on the same column are significantly different from one another ($p \le 0.05$, Duncan test).

with bradyrhizobia alone, no incompatibility between inoculants applied to the seeds was observed. Shoot dry weight was significantly increased by N fertilization and inoculations, relative to the non-inoculated control, in both sites, but shoot total N content was not statistically different in the co-inoculation treatment in Londrina, and in the single inoculation with *Bradyrhizobium* in Ponta Grossa in comparison to the non-inoculated control (**Table 2**).

Seed inoculation with *Bradyrhizobium* promoted significant increases in grain yield when compared to the non-inoculated controls without N-fertilizer in both Londrina (+4.5%) and Ponta Grossa (+10.7%), even though the soils had population of soybean bradyrhizobia (**Table 2**). However, yield of single inoculated plants was statically lower than the plants receiving 200 kg N/ha in both sites. However, seed co-inoculation with *Azospirillum* resulted in additional significant increases in grain yield in comparison to single inoculation with *Bradyrhizobium* at both sites, with yields similar to that of the N-fertilizer treatment. Compared to the non-inoculated control, overall grain yield increase due to co-inoculation was of 11.8% in Londrina and of 17.1% in Ponta Grossa (**Table 2**).

In the second year of tests, nodulation and plant growth at both locations were seriously affected by unfavorable dry weather conditions that occurred right after sowing and between sowing and the first sampling. Thereafter, weather conditions improved and rainfall became more regular, allowing plants to recover growth, reflecting in reasonable yields, although not attaining the potential expected for the region (**Table 3**). Nodule number was very low in both Rio Verde and Cachoeira Verde, attributed to the dry conditions, especially critical if we consider that the areas were cropped with soybean for the first time, showing less than 10 cells of soybean bradyrhizobia/g soil. When *Bradyrhizobium* and *Bradyrhizobium* + *Azospirillum* were applied to the seeds, significantly higher nodule biomass relative to both non-inoculated controls, either with or without N-fertilizer, could be observed in Rio Verde, and in relation to the controle with N-fertilizer in Cachoeira Dourada. However, no significant differences were observed for dry weight or total N accumulated in shoots in response to either inoculation or N fertilization (**Table 3**).

At the final harvest, in Rio Verde, but not in Cachoeira Dourada there was a slight response when N fertilizer was applied, in comparison with both the non-inoculated and the inoculated with *Bradyrhizobium* treatments (**Table 3**). At both locations, there were no significant responses to inoculation with *Bradyrhizobium*, probably due to water stress at critical phases of root infection, nodule initiation and early stages of $N_2$ fixation. Co-inoculation with *Azospirillum*, on the other hand, resulted in significant yield increases at both locations. When compared to inoculation with *Bradyrhizobium* alone, yield increases due to co-inoculation of 212 kg/ha and 565 kg/ha were observed in Rio Verde and Cachoeira Dourada, respectively. At both locations, co-inoculation treatments yielded significantly more than the non-inoculated control without N-fertilizer, and in Cachoeira-Dourada it was even superior to the N fertilized treatment with 200 kg N/ha. In comparison to the non-inoculated control without N-fertilizer, the increases due to the co-inoculation were of 7.1% in Rio Verde and of 15.4% in CachoeiraDourada (**Table 3**).

**Table 3.** Nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), total shoot N content (TSNC) at 40 days after sowing (DAS) in Rio Verde and at 50 DAS in CachoeiraDourada, and grain yield (Yield) of soybean plants (cultivars BMX-Potência (RR) and BRS-GO-8360 (conventional), respectively) in response to seed inoculation consisting of single inoculation with *Bradyrhizobium*, or of co-inoculation with *Bradyrhizobium* and *Azospirillum*. Experiments performed in 2012/13 in soybean bradyrhizobia population lower than 10 cells of soybean bradyrhizobia/g soil.

| Treatment | Rio Verde | | | | | CachoeiraDourada | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NN | NDW | SDW | TSNC | Yield | NN | NDW | SDW | TSNC | Yield |
| | (n˚/pl) | (mg/pl) | (g/pl) | (mg N/pl) | (kg/ha) | (n˚/pl) | (mg/pl) | (g/pl) | (mg N/pl) | (kg/ha) |
| Non-inoculated | 0.37 a[a] | 9.90 b | 6,92 a | 188 a | 2613 b | 1.56 a | 11.01 a | 8.43 a | 230 a | 2635 b |
| Non-inoculated + N-fertilizer (200 kg N/ha) | 0.23 a | 4.11 b | 6,53 a | 145 a | 2855 a | 0.16 a | 0.34 b | 8.41 a | 203 a | 2531 b |
| I (inoculated with *Bradyrhizobium*) | 0.73 a | 17.05 a | 7,86 a | 191 a | 2586 b | 2.20 a | 20.00 a | 9.51 a | 311 a | 2477 b |
| I + Azo (co-inoculated with *Bradyrhizobium* and *Azospirillum*) | 0.97 a | 17.83 a | 6,02 a | 130 a | 2798 a | 1.33 a | 13.07 a | 7.72 a | 204 a | 3042 a |

[a]Means (n = 6) followed by different letters on the same column are significantly different from one another ($p \le 0.05$, Duncan test).

## 4. Discussion

Several authors have addressed the issue of co-inoculating legumes with $N_2$-fixing and plant growth-promoting rhizobacteria (PGPR). There have been tests with a variety of species, e.g. alfalfa (*Medicago sativa* L.) [16] and common bean [12]. Notwithstanding, most studies have only focused the effects of the PGPR on the early aspects of the symbiotic relationship, and very few field studies. In a recent publication [12], we report the benefits of co-inoculating soybean and common bean with their respective rhizobia and *Azospirillum brasilense*, but in that case, rhizobia were applied to the seeds and *Azospirillum* was placed in the planting furrow.

We initially thought of positioning each inoculum at a different place because we believed it might be necessary to apply very large doses of *Azospirillum* that, in turn, would reduce the concentration of *Bradyrhizobium* on the seeds. However, our preliminary studies showed that larger doses of *Azospirillum* may inhibit plant growth, and that the best benefits are obtained when *Azospirilllum* inoculum size is ten times smaller than the optimal inoculum size for *Bradyrhizobium* [12]. Therefore, taking into account the concentration of the commercial products containing *Azospirillum* available in the Brazilian market, we realized it would be possible to apply both bacteria to the same seeds. In the experiments reported here, we aimed at evaluating nodulation, plant growth, and yield increases in response to soybean seed inoculation with both *Bradyrhizobium* and *Azospirillum*.

It is not clear, in the literature, whether the benefits of inoculating legumes with rhizobia and azospirilla are only due to an increase in nodulation and $N_2$ fixation, or if other indirect factors are involved. *A. brasilense* is one of the so-called PGPR, which may stimulate plant growth by means of an array of favorable mechanisms. In fact, the inoculation of common bean, in Egypt, with a mixture containing vesicular-arbuscular mycorrhizal (VAM) fungi, *Rhizobium* sp., *Azospirillum* sp., and *Bacillus circulans*, resulted in gains in plant height, branching, nodulation and plant biomass, when compared to control plants [17]. Stajković *et al.* [18] obtained similar results when they inoculated common bean with a mixture containing *Rhizobium* sp., *Pseudomonas* sp., and *Bacillus* sp. Bx, which promoted gains in plant biomass and in N and P contents in the shoots. Those authors also observed phosphate solubilization, indole-acetic acid (IAA), ammonia, and siderophore production by *Pseudomonas* sp. LG *in vitro*, what might explain common bean growth promotion. Grain yield and yield components, such as the number of pods per plant, the number of seeds per pod, the weight of 100 seeds, as well as protein content in the grains were also positively influenced by the inoculation of common bean with *Rhizobium* and *Pseudomonas fluorescens* [19]. Non-leguminous plants may also benefit from inoculation with *Azospirillum* and other $N_2$-fixing PGPR, e.g. banana (*Musa* spp.) trees present better root growth, earlier flowering, increased fruit yield, and improved fruit quality when inoculated with PGPR [20].

These reports evidence that *Azospirillum* contributes to promote plant growth and yield by means of an array of mechanisms, which, in turn, may explain our field results. *Azospirillum* is known, for instance, for improving the plant's resistance to water stress [21]. Such favorable activity might be of utmost importance to plants depending on fixed $N_2$, since it is well documented that water and temperature stresses are among the most limiting factors for biological $N_2$ fixation in the tropics, seriously compromising root infection, nodule formation, and $N_2$ fixation.

In the case of two (Rio Verde and Cachoeira Dourada) of the four experiments reported here, soybean co-inoculation with *Azospirillum* seemed to be beneficial under conditions of water stress. Our field trials revealed that the utilization of both bacteria resulted in significant average yield increase of 388 kg/ha (equivalent to 6.5 60 kg bags), or 15.3% relative to when only *Bradyrhizobium* was employed as inoculant on the seeds. Similar results were obtained when co-inoculation was compared to the non-inoculated, non-fertilized control in both experiments, and even to the N-fertilized (200 kg N/ha) control in Cachoeira Dourada (**Table 3**).

Our results demonstrate that seed co-inoculation with *Bradyrhizobium* and *Azospirillum* is agronomically efficient and is beneficial to the crop. We obtained gains in grain yields both in areas where soybean was cropped for the first time, where no soil bradyrhizobia were available, as well as in areas where the crop had been grown before, where there was already an established population of bradyrhizobia in the soil. The strategy of co-inoculation represents a new biotechnological tool to improve soybean yield without adding any chemical N fertilizers, thus contributing to current practices of sustainability in agriculture.

## 5. Conclusion

Soybean seeds can be inoculated with both *Azospirillum* and *Bradyrhizobium* inoculants, and co-inoculation improves soybean yield without the addition of any chemical N fertilizers, helping reduce costs and keeping pace with current practices of sustainability in agriculture.

## Acknowledgements

The microbiology group of Embrapa Soja is also supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), CNPq-Repensa (562008/2010-1). M. Hungria and M.A. Nogueira are also research fellows from CNPq. Approved for publication by the Editorial Board of Embrapa Soja as manuscript number 285/2014.

## References

[1] Hungria, M., Campo, R.J., Mendes, I.C. and Graham, P.H. (2006) Contribution of Biological Nitrogen Fixation to the N Nutrition of Grain Crops in the Tropics: The Success of Soybean (*Glycine max* L. Merr.) in South America. In: Singh, R.P., Shankar, N. and Jaiwa, P.K., Eds., *Nitrogen Nutrition and Sustainable Plant Productivity*, Studium Press, Houston, 43-93.

[2] Hungria, M., Franchini, J.C., Campo, R.J., Crispino, C.C., Moraes, J.Z., Sibaldelli, R.N.R., Mendes, I.C. and Arihara, J. (2006) Nitrogen Nutrition of Soybean in Brazil: Contributions of Biological $N_2$ Fixation and of N Fertilizer to Grain Yield. *Canadian Journal of Plant Sciences*, **86**, 927-939. http://dx.doi.org/10.4141/P05-098

[3] Tien, T.M., Gaskins, M.H. and Hubbell, D.H. (1979) Plant Growth Substances Produced by *Azospirillum brasilense* and Their Effect on the Growth of Pearl Millet (*Pennisetumamericanum* L.). *Applied and Environmental Microbiology*, **37**, 1016-1024.

[4] Bottini, R., Fulchieri, M., Pearce, D. and Pharis, R. (1989) Identification of Gibberelins $A_1$, $A_3$, and Iso-$A_3$ in Cultures of *A. lipoferum*. *Plant Physiology*, **90**, 45-47. http://dx.doi.org/10.1104/pp.90.1.45

[5] Strzelczyk, E., Kamper, M. and Li, C. (1994) Cytokinin-Like-Substances and Ethylene Production by *Azospirillum* in Media with Different Carbon Sources. *Microbiological Research*, **149**, 55-60. http://dx.doi.org/10.1016/S0944-5013(11)80136-9

[6] Wang, S., Huijun, W., Junqing, Q., Lingli, M., Jun, L., Yanfei, X. and Xuewen, G. (2009) Molecular Mechanism of Plant Growth Promotion and Induced Systemic Resistance to Tobacco Mosaic Virus by *Bacillus* spp. *Journal of Microbiology and Biotechnology*, **19**, 1250-1258. http://dx.doi.org/10.4014/jmb.0901.008

[7] Rodriguez, H., Gonzalez, T., Goire, I. and Bashan, Y. (2004) Gluconic Acid Production and Phosphate Solubilization by the Plant Growth-Promoting Bacterium *Azospirillum* spp. *Naturwissenschaften*, **91**, 552-555. http://dx.doi.org/10.1007/s00114-004-0566-0

[8] Döbereiner, J. and Pedrosa, F.O. (1987) Nitrogen-Fixing Bacteria in Non-Leguminous Crop Plants. Science Tech, Springer Verlag, Madison.

[9] Hungria, M., Campo, R.J., Souza, E.M. and Pedrosa, F.O. (2010) Inoculation with Selected Strains of *Azospirillumbrasilense* and *A. lipoferum* Improves Yields of Maize and Wheat in Brazil. *Plant and Soil*, **331**, 413-425.

http://dx.doi.org/10.1007/s11104-009-0262-0

[10] Marks, B.B., Megías, M., Nogueira, M.A. and Hungria, M. (2013) Biotechnological Potential of Rhizobial Metabolites to Enhance the Performance of *Bradyrhizobium japonicum* and *Azospirillum brasilense* Inoculants with the Soybean and Maize Crops. *Applied Microbiology and Biotechnology Express*, **3**, 21.

[11] Campo, R.J., Araujo, R.S. and Hungria, M. (2009) Nitrogen Fixation with the Soybean Crop in Brazil: Compatibility between Seed Treatment with Fungicides and Bradyrhizobial Inoculants. *Symbiosis*, **48**, 154-163. http://dx.doi.org/10.1007/BF03179994

[12] Hungria, M., Nogueira, M.A. and Araujo, R.S. (2013) Co-Inoculation of Soybeans and Common Beans with Rhizobia and Azospirilla: Strategies to Improve Sustainability. *Biology and Fertility of Soils*, **49**, 791-801. http://dx.doi.org/10.1007/s00374-012-0771-5

[13] MAPA (Ministério da Agricultura, Pecuária e Abastecimento) (2010) Instrução Normativa Nº 30, de 12/11/2010. http://sistemasweb.agricultura.gov.br/sislegis/loginAction.do?method=exibirTela

[14] Cassán, F., Penna, C., Creus, C., Radovancich, D., Monteleleone, E., Salamone, I.G., Salvo, L.D., Mentel, I., Garcia, J., Pasarello, M.C.M., Ltt, L., Puente, M., Correa, O., Puunschke Valerio, K., Massa, R., Roosi, A., Diaz, M., Catafesta, M., Righes, S., Carletti, S. and Cáceres, E.R. (2010) Protocolo para el control de calidad de inoculantes que contienen *Azospirillum* sp. Associación Argentina de Microbiología, Buenos Aires. (Documento de Procedimientos de la REDCAI, 2)

[15] MAPA (Ministério da Agricultura, Pecuária e Abastecimento) (2011) Instrução Normativa Nº 13, de 24/03/2011. http://sistemasweb.agricultura.gov.br/sislegis/loginAction.do?method=exibirTela

[16] Itzigsohn, R., Kapulnik, Y., Okon, Y. and Dovrat, A. (1993) Physiological and Morphological Aspects of Interactions between *Rhizobium meliloti* and Alfalfa (*Medicago sativa*) in Association with *Azospirillum brasilense*. *Canadian Journal of Microbiology*, **39**, 610-615. http://dx.doi.org/10.1139/m93-088

[17] Massoud, O.N., Morsy, E.M. and El-Batanony, N.H. (2009) Field Response of Snap Bean (*Phaseolus vulgaris* L.) to $N_2$-Fixers *Bacillus circulans* and *Arbuscular mycorrhizal* Fungi Inoculation through Accelerating Rock Phosphate and Feldspar Weathering. *Australian Journal of Basic and Applied Sciences*, **3**, 844-852.

[18] Stajković, O., Delić1, D., Jošić, D., Kuzmanović, Đ., Rasulić, N. and Knežević-Vukčević, J. (2011) Improvement of Common Bean Growth by Co-Inoculation with *Rhizobium* and Plant Growth-Promoting Bacteria. *Romanian Biotechnological Letters*, **16**, 5919-5926.

[19] Yadegari, M. and Asadi Rahmani, H. (2008) Evaluation of Bean (*Phaseolus vulgaris)* Seeds Inoculation with *Rhizobium phaseoli* and Plant Growth Promoting Rhizobacteria(PGPR) on Yield and Yield Components. *African Journal of Agricultural Research*, **5**, 792-799.

[20] Baset Mia, M.A., Shamsuddin, Z.H. and Mahmood, M. (2010) Use of Plant Growth Promoting Bacteria in Banana: A New Insight for Sustainable Banana Production. *International Journal of Agriculture and Biology*, **12**, 459-467.

[21] Cassán, F.D. and Garcia De Salomone, I., Eds. (2008) *Azospirillum* sp.: Cell Physiology, Plant Interactions and Agronomic Research in Argentina. Associación Argentina de Microbiología, Buenos Aires.